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Both Myoblast Lineage and Innervation Determine Expression of the Slow Myosin Heavy Chain 2 Gene

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Skeletal muscle fibers express members of the myosin heavy chain (MyHC) gene family in a fiber-type-specific manner. In avian skeletal muscle it is the expression of the slow MyHC isoforms that most clearly distinguishes slow- from fast-contracting fiber types. Two hypotheses have been proposed to explain fiber-type-specific expression of distinct MyHC genes during development—an intrinsic mechanism based on the formation of different myogenic lineage(s) and an extrinsic, innervation-dependent mechanism. We developed a cell culture model system in which both mechanisms were evaluated during fetal muscle development. Myoblasts isolated from prospective fast (pectoralis major) or slow (medial adductor) fetal chick muscles formed muscle fibers in cell culture, none of which expressed slow MyHC genes. By contrast, when muscle fibers formed from myoblasts derived from the slow muscle were cocultured with neural tube, the muscle fibers expressed a slow MyHC gene, while muscle fibers formed from myoblasts of fast muscle origin continued to express only fast MyHC. Motor endplates formed on the fibers derived from myoblasts of both fast and slow muscle origin in cocultures, and slow MyHC gene expression did not occur when neuromuscular transmission or depolarization was blocked. We have cloned the slow MyHC gene that is expressed in response to innervation and identified it as the slow MyHC 2 gene, the predominant adult slow isoform. cDNAs encoding portions of the three slow myosin heavy chain genes (MyHC1, slow MyHC 2, and slow MyHC 3) were isolated. Only slow MyHC 2 mRNA was demonstrated to be abundant in the cocultures of neural tube and muscle fibers derived from myoblasts of slow muscle origin. Thus, expression of the slow MyHC 2 gene in this *in vitro* system indicates that formation of slow muscle fiber types is dependent on both myoblast lineage (intrinsic mechanisms) and innervation (extrinsic mechanisms), and suggests neither mechanism alone is sufficient to explain formation of muscle fibers of different types during fetal development. © 1997 Academic Press

INTRODUCTION

Vertebrate skeletal muscles are composed of muscle fibers formed from muscle precursor cells, called myoblasts. Within each muscle fiber a number of muscle-specific contractile proteins are synthesized from families of genes encoding multiple protein isoforms. The combinations of isoform genes expressed in particular muscle fibers are large and diverse (Staron and Pette, 1987) giving rise to a multiplicity of muscle fiber phenotypes with unique repertoires of muscle-specific proteins and associated physiological characteristics. In particular, expression of members of the myosin heavy chain (MyHC) multigene family defines fiber type identity and significantly affects fiber contractile properties via myosin ATPase activity (Reiser *et al.*, 1988a,b). In broad terms, fibers are classified as fast, fast/slow (mixed), or slow depending on the presence of MyHC isoforms with fast and/or slow ATPase activities. A central issue in myogenesis is what determines the formation of different muscle fiber types (Stockdale, 1997).

Members of both fast and slow MyHC gene subfamilies are expressed in developmental and tissue-specific patterns. Transitions in fast MyHC isoforms occur in nearly all avian skeletal muscle fibers. These transitions typically involve successive expression of embryonic and then neonatal and, finally, adult fast MyHC genes (Whalen *et al.*, 1981; Crow and Stockdale, 1986a; Bandman and Bennett, 1988). However, the final fast MyHC genes expressed in adult muscle

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fibers vary (Crow and Stockdale, 1986a). The adult chicken pectoralis major (PM), for example, expresses a unique fast MyHC gene, whereas the adult biceps brachii exclusively express the embryonic fast MyHC gene (Bandman and Bennett, 1988). In contrast, changes in the expression of slow isoforms of MyHC during muscle development are more limited and less variant (Page *et al.*, 1992; Nikovits *et al.*, 1996).

Mechanisms that establish these differential patterns of MyHC gene expression and, therefore, fiber-type identity are operative from the outset of muscle formation. In avian limbs primary muscle fibers begin forming between Embryonic Days 4 and 5 (ED 4 and 5) giving rise to dorsal and ventral premuscle masses, and by ED 6 separate anatomic muscles are present. Based on expression of specific MyHC isoforms, distinct fiber types exist among the primary fibers in the premuscle masses as early as ED 5 (Page *et al.*, 1992) and are clearly localized within specific anatomic muscles at ED 6 (Crow and Stockdale, 1986b). Therefore, differences in muscle fiber phenotype are evident as fibers first form, and before muscle fibers are first innervated *in vivo* at ED 5–ED $5^{1/2}$ (HH stages 27–28) (Hamburger and Hamilton, 1951; Landmesser and Morris, 1975; Lance-Jones and Landmesser, 1980, 1981). As primary fiber formation wanes, a second population of myogenic precursor cells, fetal myoblasts, arise and form secondary, or fetal, muscle fibers in apposition with the primary fibers (Duxson *et al.*, 1989). Here too, differential expression of MyHC genes generates diversity in muscle fiber phenotype *in vivo* (Crow and Stockdale, 1986a; Bandman and Bennett, 1988; Condon *et al.*, 1990a). For example, primary and secondary fibers of the chicken superficial biceps and PM muscles exclusively express fast MyHC isoforms, while all the secondary fibers of the medial adductor (MA) muscle express members of both fast and slow classes of MyHCs (Page *et al.*, 1992).

Studies suggest that intrinsic as well as extrinsic mechanisms play a role in primary and secondary fiber typing. Muscle fibers formed *in vitro* from cloned embryonic chick myoblasts demonstrate an intrinsic control of MyHC gene expression. Colonies of differentiated muscle fibers formed from clonal populations of individual embryonic myoblasts either express fast MyHC genes exclusively or express both fast and slow MyHC genes (Miller and Stockdale, 1986a,b). Intrinsic differences in myoblast response to growth factors (Cusella-De Angelis *et al.*, 1994; Seed and Hauschka, 1988) and in expression of additional contractile protein genes have also been demonstrated when myoblasts from fetal and adult stages of development form fibers *in vitro* (Hartley *et al.*, 1991; Feldman and Stockdale, 1991; Pin and Merrifield, 1993; Düsterhöft and Pette, 1993). Intrinsic mechanisms of embryonic muscle fiber type formation have been examined *in vivo* as well, by reintroduction of cloned myoblasts into embryonic chick limbs. Cloned myoblasts injected into embryonic limb buds differentiated *in vivo* forming fibers that expressed the same MyHC genes which they would have had these same myoblasts differentiated in cell culture (DiMario *et al.*, 1993). Similar results have been reported for mammalian myoblasts injected into rat muscles (Pin and Merrifield, 1997). However, apparently different observations, possibly the result of differences in experimental design, have led some to question the role of intrinsic mechanisms in establishing fiber type heterogeneity (Cho *et al.*, 1993; Hughes and Blau, 1992). The latter studies suggest that extrinsic mechanisms are solely responsible for differential MyHC gene expression and diversity of fiber types.

The importance of extrinsic determinants of fiber type is most evident in denervation experiments during fetal muscle development. Most studies have shown that functional denervation with curare (Crow and Stockdale, 1986b), β-bungarotoxin (Condon *et al.*, 1990b), or nerve ablation (Butler *et al.*, 1982; Phillips and Bennett, 1984) has little effect on the formation or persistence of primary muscle fiber types in the early embryo. Furthermore, denervated primary muscle fibers expressed fast and slow MyHC genes in the same patterns and relative fiber numbers when compared to innervated muscles suggesting that primary myogenesis *in vivo*, as *in vitro*, is innervation independent (Stockdale, 1992; see Fredette and Landmesser, 1991, for differing results). In contrast, denervation studies during fetal secondary myogenesis have shown that muscle fiber formation and/or maintenance is dependent on innervation *in vivo* (Condon *et al.*, 1990b; Harris, 1981; McLennan, 1983; Ross *et al.*, 1987). Expression of slow MyHC genes was altered by surgical denervation, or functional denervation (Crow and Stockdale, 1986b), of secondary fibers *in vivo* and fewer secondary fibers formed in denervated limbs. Specifically, MyHC 2 expression was delayed or absent in denervated quail slow muscle *in vivo* (Lefeuvre *et al.*, 1996).

Cross-reinnervation studies also support the contention that extrinsic factors affect fiber type. Fast muscle fibers reinnervated by nerves that normally innervate slow fibers induced fast fibers to express slow MyHC(s) and cross-reinnervated slow fibers expressed fast MyHC(s) (Gauthier *et al.*, 1983; Pette and Vrbova, 1985). Interestingly, however, conversion of slow to fast MyHC gene expression in cross-reinnervated slow fibers was not complete. In sum, experimental work demonstrates that MyHC gene expression especially during fetal development is controlled by innervation-dependent as well as innervation-independent (intrinsic) mechanisms (Hoh and Hughes, 1988; Stockdale, 1992).

To examine intrinsic and extrinsic mechanisms that regulate MyHC gene expression and fiber type identity, we have developed an *in vitro* model system in which to test the roles of both myoblast lineage and innervation in the formation of different muscle fiber types. In this culture system formation of diverse muscle fiber types by differential MyHC gene expression recapitulates secondary muscle fiber formation found *in vivo*. We find that the type of fiber formed following innervation *in vitro* is dependent on the lineage of myoblasts from

which the fiber formed. By cloning the myosin heavy chain gene that most clearly defines avian slow fiber types in development and in the adult, the slow MyHC 2 gene, we have shown that it is the expression of this gene that distinguishes the response to innervation of fibers formed in cell culture from myoblasts of fast or slow muscle origin. Expression of this gene in the culture system presented here demonstrates that diversity in muscle fibers types is regulated by both intrinsic (lineage) and extrinsic (innervation) mechanisms.

MATERIALS AND METHODS

Cell Culture

Fetal myoblasts from ED 12 chick MA and PM muscles were isolated and cultured (4×10^5 myoblasts/35-mm collagen-coated dish) as previously described (Miller and Stockdale, 1986b; O'Neill and Stockdale, 1972). On Day 3 of culture, spinal cords were dissected from the thoracic and lumbar regions of ED 5 chick embryos and placed into Hanks' balanced salt solution. Care was taken to remove somitic tissue. Spinal cord segments of approximately 1 mm in length were added to each dish with the equivalent of four spinal cords placed into each 35-mm culture dish of differentiated muscle fibers. No attempt was made to keep spinal cord segments from the thoracic and lumbar regions separate. Cultures contained thoracic or lumbar segments at random. Within 24 hr, the spinal cord explants extended multiple processes and attached to the collagen-coated culture dishes. Medium consisting of 15% horse serum (Gibco/BRL), 5% chick embryo extract, in Ham's F-10 basal medium supplemented with 1.1 m*M* $CaCl_2$ and antibiotics (Gibco/BRL; penicillin/streptomycin/Fungizone) was changed every 2 days. For some cultures, 10 μM tetrodotoxin or 16 μM *d*-tubocurarine (Sigma) was added to the medium at the same time as spinal cord explants.

Immunocytochemistry

Muscle fibers were immunostained for fast and slow MyHCs with monoclonal antibodies F59 and S58, respectively. The specificities of these antibodies have been previously described (Crow and Stockdale, 1986a; Page *et al.*, 1992). Briefly, mAb F59 is an IgG 1 that recognizes multiple fast MyHC isoforms and immunostains all avian muscle fibers *in vivo* and *in vitro.* MAb S58 is an IgA that recognizes slow MyHC 2 and 3. Cultures were washed twice with phosphate-buffered saline and then fixed for 5 min with 100% ethanol. Cultures were washed again with PBS. Blocking solution consisting of 5% horse serum and 2% bovine serum albumin in PBS was added to the cultures for 1 hr at room temperature. mAb F59 and S58 supernatants were diluted 1:10 in blocking solution and then added to the cultures for 1 hr at room temperature. Cultures were washed with PBS. Fluorescein-conjugated anti-mouse IgA (Zymed) and Texas red-conjugated anti-mouse IgG (Vector) were diluted 1:50 and 1:100, respectively, in blocking solution and added to the culture dishes for 1 hr. Cultures were washed as previously and a drop of 2.5% diazabicyclooctane in 90% glycerol was added before viewing the cultures by epifluorescence. Acetylcholine receptor (AchR) clusters were visualized by incubation of the cultures in medium containing 100 n*M* rhodamine-conjugated α-bungarotoxin (Molecular Probes, Inc.) for 1 hr at 37°C prior to fixation for MyHC immunostaining.

Construction of cDNA Library and cDNA Cloning

Poly(A)$^+$ RNA was isolated from quail MA muscle using an mRNA purification kit (Pharmacia). cDNA was synthesized by random priming, size fractionated, and ligated into λgt10 vector according to manufacturer's instructions (GIBCO BRL; SuperScript Choice System). Recombinant phage DNA was packaged into phage particles according to manufacturer's instructions (Stratagene; Gigapack II Plus Packaging Extract). Phage stock titer was amplified before screening. Recombinant phage plaques (6.0×10^5) were grown overnight using P2392 host bacteria (Stratagene). Plaques were blotted onto duplicate 132-mm nitrocellulose circles (Schleicher and Scheull; BA85) for 2 min and air-dried. Blots were placed onto 8-ml pools of 0.2 *M* NaOH, 1.5 *M* NaCl twice for 5 min each and then onto 8-ml pools of 0.4 *M* Tris, 2× SSC, pH 7.6, and finally 2× SSC for 5 min each. Blots were air-dried and baked at 80°C for 2 hr in a vacuum. Blots were prehybridized in 6× SSC, 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 0.5% SDS for 4 hr at 42°C. A 562-bp cDNA fragment encoding part of slow MyHC 1 was labeled by random priming (Pharmacia; Oligolabelling Kit) with [α-^{32}P]dCTP (Amersham). This probe (200 ng) was denatured by boiling for 5 min and added (10^7 cpm/10 ml hybridization solution) to the prehybridization solution. Blots were hybridized overnight at 42°C. Blots were subsequently washed in 500 ml of 0.1× SSC, 0.01% SDS at 60°C four times for 30 min each. Blots were air-dried and used to expose Kodak X-OMAT AR film at −80°C for 1 week. Two positive plaques were picked and screened as before until all plaques hybridized to the 562-bp cDNA probe. Phage clones were amplified and DNA was isolated (Qiagen; Lambda DNA Isolation Kit). Phage clone DNA was sequenced by cycle-sequencing (GIBCO BRL). DNA analysis was carried out using Genetics Computer Group (GCG) DNA analysis software.

Northern Blot Analysis and RNA Quantitation

Total RNA was extracted (TEL-TEST "B," Inc.; RNA Stat-60) from tissues and Day 10 cultures and electrophoresed in a 1% agarose/formaldehyde gel. RNA was transferred to nitrocellulose (Schleicher and Schuell; BA-85) by capillary action (Sambrook *et al.*, 1989), baked for 2 hr at 80°C in a vacuum and prehybridized for 4 hr in 20 ml of 6× SSC, 5× Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate, and 0.1 mg/ml salmon sperm DNA at 42°C. Blots were hybridized with ^{32}P-end-labeled oligonucleotides (10^7 cpm/10 ml hybridization solution) overnight in the same prehybridization solution at 42°C. Oligonucleotides 15NC (5′-TGC TGC ATG ATC TGG TCC TCC C-3′), MHC11 oligo (5′-GAA GGA GGA TGA GCT GCA GCC C-3′), and 145PN2 (5′-CCT GAA GCT GAT GTG CAC CCC CAA A-3′) were generated from isoform-specific sequence encoding avian slow MyHCs 1, 2, and 3, respectively. Blots were washed four times for 30 min each in 250 ml of 1× SSC, 0.05% sodium pyrophosphate at 55°C. Kodak X-OMAT AR film was exposed overnight at −80°C with an intensifying screen. For quantitation, serial dilutions from 1 to 5 μg of total RNA were applied to nitrocellulose in a slot-blot apparatus and hybridized to MHC11 oligo as above. Quantitation of slow MyHC 2 RNA was performed with an LKB Ultrascan densitometer.

RESULTS

Chick Myoblasts of Fast and Slow Muscle Origin Form Exclusively Fast Fibers in Cell Culture

All MA muscle fibers of the fetal chick express both fast and slow MyHC genes during development. Both slow

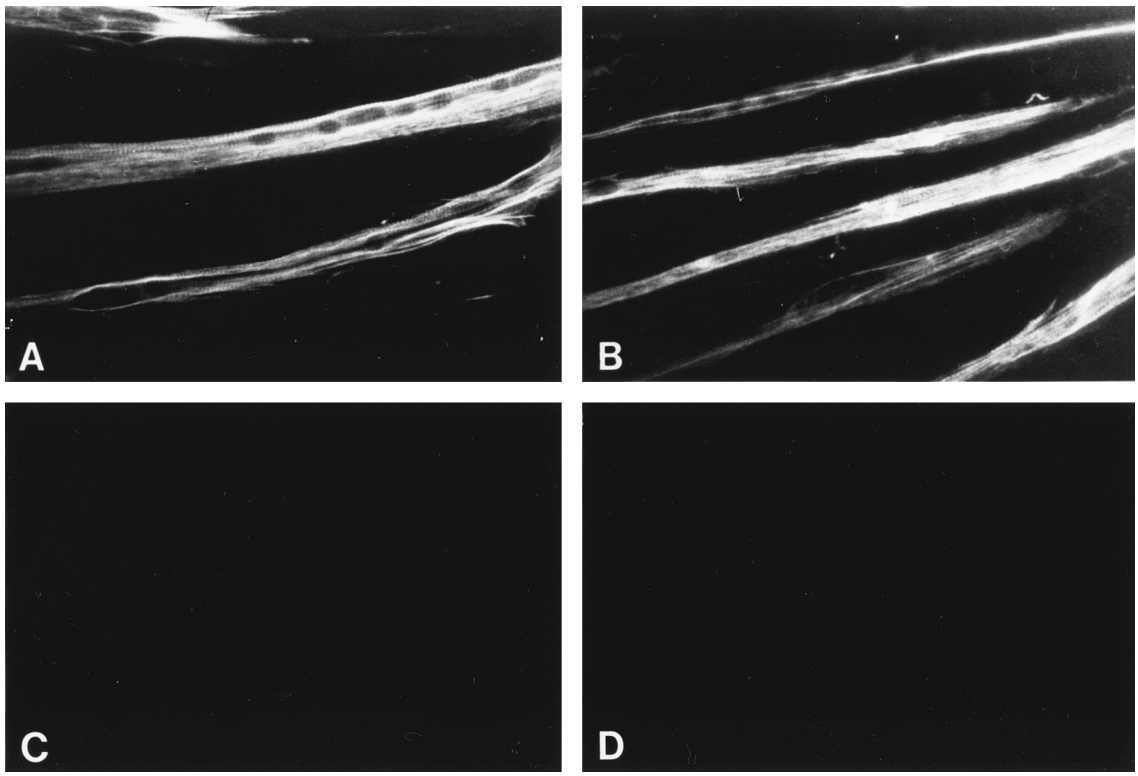

FIG. 1. Fibers formed from myoblasts of either fast or slow muscle origin express only fast MyHC isoforms in the absence of nerves. Myoblasts isolated from fetal (ED 12) chick pectoralis major (fast muscle) or medial adductor (slow muscle) were cultured for 10 days, and the muscle fibers that formed were double stained with monoclonal antibodies F59 (A, B) and S58 (C, D) which recognize fast and slow MyHC epitopes, respectively. Muscle fibers formed from medial adductor (A, C) and pectoralis major (B, D) stained for fast MyHC isoforms (A, B) but not for slow MyHC isoforms (C, D).

MyHCs 1 and 2 are present in MA muscle fibers at all stages of development (Page *et al.*, 1992). The fetal chick PM expresses principally fast MyHC isoforms (Crow and Stockdale, 1986a, Bandman and Bennett, 1988). To determine whether these patterns of MyHC gene expression are retained *in vitro*, myoblasts were isolated from the MA (slow muscle) or PM (fast muscle) muscles of ED 12 chick fetuses and cultured for 10 days. By the third day of incubation numerous multinucleated muscle fibers had formed throughout the dishes. There were no morphological differences in the appearance of the fibers formed from either source. At Day 10 of incubation, muscle fibers were double immunostained with monoclonal antibodies F59 and S58 to detect fast and slow MyHC isoforms, respectively (Fig. 1). Muscle fibers derived from fetal chick MA or PM myoblasts expressed a fast MyHC(s) as shown by F59 immunostaining (Figs. 1A and 1B), but did not express a slow MyHC as shown by the lack of S58 immunostaining (cf. Figs. 1A and 1B with 1C and 1D). To confirm that myoblasts isolated from fetal MA muscle do not form slow muscle fibers in culture, 481 myoblasts were cloned and the fibers formed in each colony were assessed by immunostaining with S58. None of these clones stained positively for slow MyHC isoforms (data not shown). These results indicate that fetal myoblasts of fast or slow muscle origin, when cultured alone, formed exclusively fast muscle fibers.

Myoblasts of Slow but Not Fast Muscle Origin Form Fibers That Express Slow MyHC When Cocultured with Segments of Spinal Cord

Cocultures of fetal myoblasts and embryonic spinal cord segments were established to determine whether innervation of differentiated muscle fibers affects MyHC gene expression *in vitro*. Fetal myoblasts from ED 12 chick MA and PM muscles were isolated and cultured as above, except that on the third day of incubation, spinal cord segments from the thoracic and lumbar regions of ED 5 chick embryos were placed into the cultures. By ED 5, motor neurons are located in the ventral neural tube (Tanaka and Obata, 1984). Care was taken to remove somitic tissue, and controls of spinal cord explants alone were set. By Day 10 of incubation, multiple cell processes from the spinal cord explants extended throughout the cultures and attached to the collagen substrate and muscle fibers. These cocultures were stained with F59 and S58 (Fig. 2). Cocultures containing fibers formed from myoblasts of the fetal PM stained only with F59 (Figs. 2B and 2D). Cocultures of fibers formed from

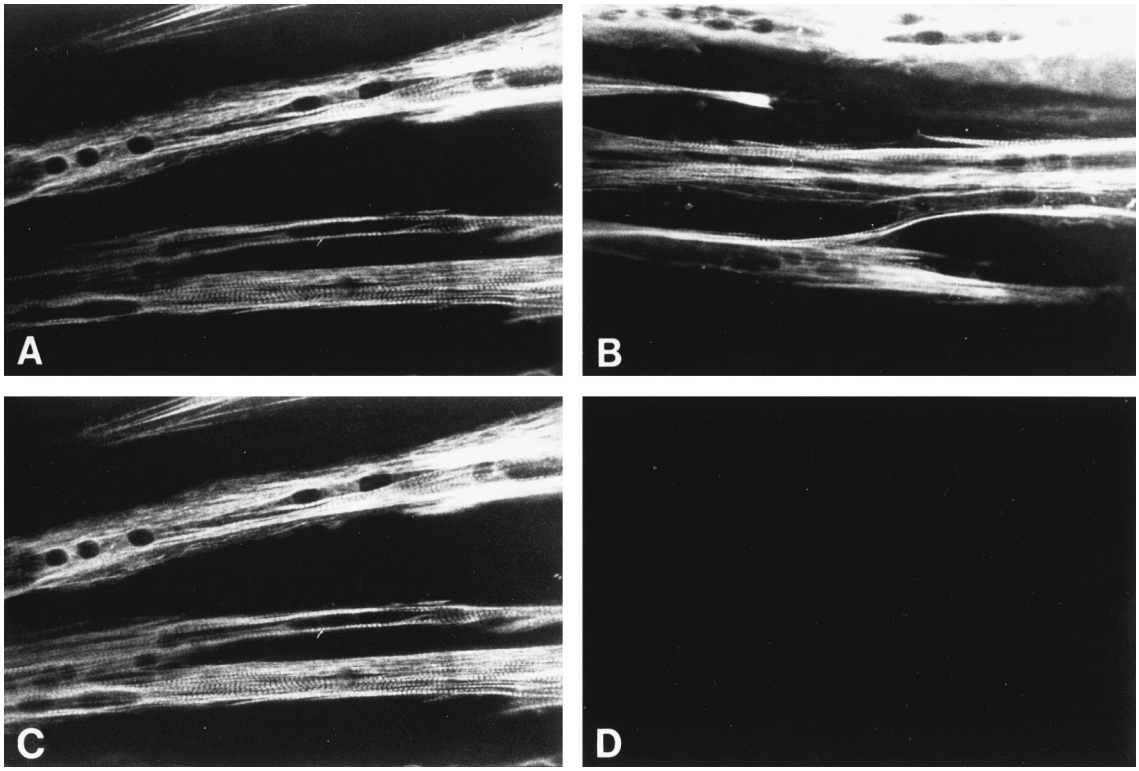

FIG. 2. Spinal cord explants initiate expression of slow MyHC expression in muscle fibers derived from myoblasts from fetal medial adductor but not from pectoralis major muscle. Myoblasts were isolated from fetal medial adductor or pectoralis major (ED 12) and cultured for 10 days. On the third day of culture, spinal cord explants from ED 5 chick embryos were placed into the cultures. Muscle fibers were double stained with F59 (A, B) and S58 (C, D). All muscle fibers formed from medial adductor (A, C) or pectoralis major (B, D) myoblasts stained for fast MyHC isoforms (A, B). Only muscle fibers formed from medial adductor myoblasts and cocultured with spinal cord explants stained for slow MyHC isoforms (C). Muscle fibers formed from pectoralis myoblasts and cocultured with spinal cord explants did not express slow MyHC isoforms (D).

fetal MA myoblasts stained with both F59 and S58 (Figs. 2A and 2C). Slow MyHC staining with S58 occurred only within the MA cultures in which spinal cord was added. Immunostaining for slow MyHC in these cultures did not extend to all the muscle fibers, but was detected in all muscle fibers near a spinal cord explant. Examination of the slow myosin staining demonstrated uniform distribution of the protein throughout these fibers whether or not sarcomeres could be discerned. In those fibers with distinct sarcomeres, slow MyHC was localized to all sarcomeres throughout the muscle fibers. Control dishes of spinal cord explants alone did not contain muscle fibers nor did they immunostain with F59 or S58 (data not shown). Thus, myoblasts from fetal fast and slow muscles formed muscle fibers that expressed only fast MyHC isoforms when cultured in the absence of spinal cord. However, only fibers derived from MA myoblasts expressed slow MyHC isoforms in the presence of spinal cord neurites.

Isolation of cDNAs Encoding Slow Myosin Heavy Chains 1 and 2

Based on characterization of protein isoforms, three slow MyHCs, slow MyHCs 1, 2, and 3, have been identified in avian skeletal muscle (Kennedy *et al.*, 1986; Hoh *et al.*, 1976; Crow and Stockdale, 1986b; Page *et al.*, 1992). Recently the gene for slow MyHC 3 has been cloned and sequenced (Nikovits *et al.*, 1996), but until this report slow MyHC 1 and slow MyHC 2 cDNAs have not been cloned. *In vivo*, slow muscles such as the MA and anterior latissimus dorsi (ALD) express one or more of these slow MyHCs during the course of development. PAGE analysis has shown that the predominant isoform in the ALD during fetal development is slow MyHC 1 and as development proceeds, it is downregulated as slow MyHC 2 is upregulated (Kennedy *et al.*, 1986). The high degree of primary sequence homology among MyHC proteins in general, and among slow MyHC proteins in particular, has made development of isoform-specific antibodies problematic. In particular, mAb S58 recognizes both slow MyHCs 2 and 3 (Page *et al.*, 1992). Therefore, to identify the slow MyHC gene expressed in response to innervation in our cocultures, we have generated cDNA probes specific for each of the three known slow MyHC isoforms.

Both the full-length cDNA and the gene for slow MyHC 3 have been previously sequenced, providing slow MyHC

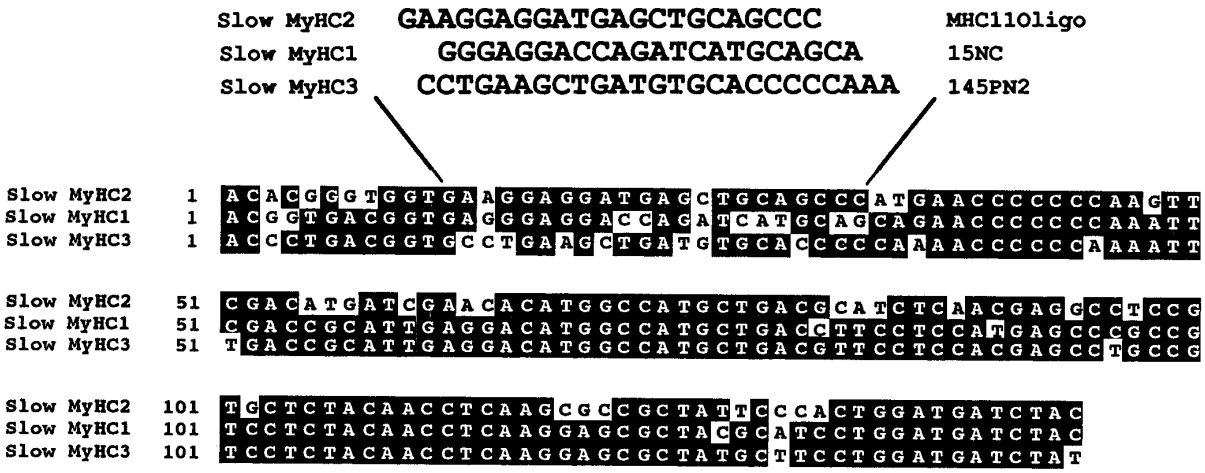

FIG. 3. Sequence comparison of slow MyHC cDNAs used to generate isoform-specific probes. Sequence of the slow MyHC 3 (Nikovits *et al.*, 1996) and partial cDNAs encoding slow MyHCs 1 and 2 are shown with maximal alignment. Sequence near the 5′ end of slow MyHC 3 exon 4 and within the homologous regions of the other cDNAs provided sufficient sequence divergence to generate isoform-specific oligonucleotide probes. The oligonucleotides generated to each cDNA are shown above the cDNA sequences for slow MyHC 1, 2, and 3.

3-specific probes (Nikovits *et al.*, 1996); however, specific probes for slow MyHCs 1 and 2 did not exist. Our initial effort focused on isolating cDNA clones of slow MyHC 1. During the isolation of the slow MyHC 3 gene, a 562-bp cDNA clone, designated MHC15, was isolated and sequenced. While clearly different in sequence from slow MyHC 3 cDNA, MHC15 had greater sequence homology to slow MyHC 3 DNA than to any other MyHC sequence. To identify the pattern of expression of the gene that encodes MHC15 and distinguishes it from slow MyHC 3, isoform-specific oligonucleotides, 145PN2 and 15NC, for slow MyHC 3 and the MHC15 isoform, respectively, were generated from highly divergent sequence near the 5′ end of the slow MyHC 3 gene (Fig. 3). The specificity of each oligonucleotide probe was shown by hybridization to a panel of RNAs isolated from adult liver, the adult PM, which exclusively expresses fast MyHC genes, the Day 1 posthatch (PH 1) MA muscle which expresses fast and slow MyHCs 1 and 2, the PH 1 atria which expresses the slow MyHC 3 gene (Nikovits *et al.*, 1996), and embryonic, fetal, and adult ALD muscle in which slow MyHC 1 gene expression has been shown to be downregulated, and slow MyHC 2 is upregulated during development (Kennedy *et al.*, 1986). Northern blot analysis of RNA isolated from developing slow muscles using the oligonucleotide 15NC from the MHC15 cDNA revealed a pattern of expression most consistent with slow MyHC 1 (Fig. 4). That the MHC15 cDNA did not encode slow MyHC 3 was indicated by the lack of hybridization to RNA from the atria which strongly expresses slow MyHC 3 (Wang *et al.*, 1996; Nikovits *et al.*, 1996; Page *et al.*, 1992). It did not hybridize to fast muscle RNA. 15NC hybridized to RNAs from the slow MA muscle and the slow ALD muscle, particularly at fetal and early stages with a signal that is greater at the early stages than at the later stages of development of these slow muscles. A pattern in which slow myosin heavy chain expression is at higher levels at fetal periods and decreases as development proceeds matches that reported for slow MyHC 1 protein expression in these muscles (Kennedy *et al.*, 1986). We conclude that the MCH15 cDNA encodes slow MyHC 1. This conclusion was validated by cloning a slow MyHC cDNA that encodes slow MyHC 2.

To obtain a cDNA encoding slow MyHC 2 a cDNA library was constructed from fetal quail MA poly(A)$^+$ RNA and screened with the 562-bp MHC15 cDNA. At fetal stages of development the MA expresses all three slow MyHCs (Page *et al.*, 1992). A clone was isolated that contained a 1803-bp insert with a sequence significantly different from MHC15 (75.2% identity), or to slow MyHC 3 cDNA (69.4% identity). This clone was designated MHC11 and an oligonucleotide probe (MHC11 oligo) was generated from sequence which had relatively poor sequence identity with the same region of slow MyHC 3 and slow MyHC 1 (Fig. 3). On Northern blots (Fig. 4), this oligonucleotide hybridized in a pattern identical to the pattern of slow MyHC 2 protein expression (Kennedy *et al.*, 1986); i.e., the signal increased with slow muscle development as the signal for slow MyHC 1 decreased (Fig. 4). This Northern analysis shows that an mRNA that hybridizes with MHC11 cDNA is present exclusively in slow skeletal muscles such as the MA and ALD and supplants slow MyHC 1 mRNA to become the predominant form in the adult ALD. No significant levels of hybridization to mRNAs from the adult liver, atria, or fast PM muscle were detected. Therefore, cDNA MHC11 encodes the slow MyHC 2 isoform.

Analysis of sequence contained within the slow MyHC 2 cDNA clone demonstrates that it encodes the amino terminal portion of slow MyHC 2 (Fig. 5). This cDNA contains 74 bp of 5′ untranslated sequence followed by a 1729-bp

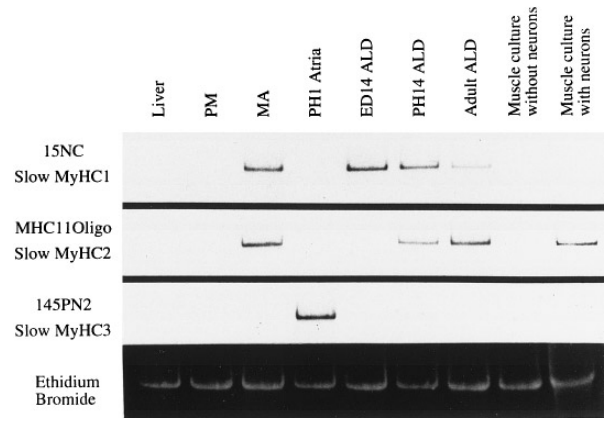

FIG. 4. Northern analysis identifies cDNA clones encoding slow MyHCs 1, 2, and 3 and demonstrates slow MyHC 2 gene expression in muscle fibers formed from medial adductor myoblasts in the presence of spinal cord. Total RNA was isolated from tissues (adult liver, adult pectoralis major (PM), Day 1 posthatch (PH1) medial adductor (MA), PH 1 atria, and anterior latissimus dorsi (ALD) from Embryonic Day 14 (ED 14), Day 14 posthatch (PH 14), and adult stages), and from muscle fibers formed from fetal chick medial adductor myoblasts cultured with and without spinal cord explants. RNA (5 μg) was electrophoresed, blotted, and hybridized with oligonucleotides specific for slow MyHC 1 (15NC), slow MyHC 2 (MHC11 oligo), or slow MyHC 3 (145PN2). Equivalent RNA loading in each lane was demonstrated by ethidium bromide staining of the 18S ribosomal subunit. Hybridization patterns of probes 15NC (slow MyHC 1) and MHC11 oligo (slow MyHC 2) generated from the newly cloned cDNAs are identical to patterns of protein expression (Kennedy *et al.*, 1986) for slow MyHCs 1 and 2. RNA isolated from muscle fiber/spinal cord cocultures hybridized to MHC11 oligo demonstrating that the slow MyHC gene expressed in these cultures is the slow MyHC 2, whereas muscle fibers cultured alone did not express the slow MyHC 2 gene at detectable levels.

open reading frame encoding 576 amino acids. Three possible initiating methionines are among the first six amino acid residues. A comparison of amino acid sequences from several MyHCs shows regions of sequence divergence and regions of conservation (Fig. 6). The amino termini of these MyHCs contain highly divergent amino acid sequence. Sequence identities between slow MyHC 2 and the other MyHCs range from 20 to 36% over the amino terminal 70 residues, whereas in the next 70 residues, sequence identity between slow MyHC 2 and the other MyHCs increases to 63–69%. The slow MyHC 2 amino acid sequence is most similar (54.3%) to the rat β-cardiac/slow MyHC amino acid sequence over the amino terminal 140 residues.

Muscle Fibers in Coculture Express the Slow MyHC 2 Gene

While immunoblotting identified a slow MyHC isoform in MA cultures that contained neural tube, the recognized cross-reactivity of monoclonal antibodies to MyHCs that share close sequence homology required more specific identification. To unambiguously determine which slow MyHC isoform gene(s) was expressed in direct response to innervation, oligonucleotides specific for each of the three known slow MyHC genes were used to probe Northern blots of total RNAs isolated from several control tissues, and cultures with and without spinal cord explants (Fig. 4). Cocultures of spinal cord explants and muscle fibers formed from MA myoblasts expressed the slow MyHC 2 gene as shown by hybridization with MHC11 oligonucleotide. The cocultures did not express significant levels of either slow MyHC 1 or slow MyHC 3. These results demonstrate that it is the slow MyHC 2 gene that is expressed in response to coculture with spinal cord explants. Densitometric analysis of hybridization to the RNA isolated from MA muscle fibers cocultured with spinal cord explants demonstrated that expression of the slow MyHC 2 gene was increased approximately eightfold relative to MA muscle fibers cultured without spinal cord explants.

The Lack of MyHC 2 Gene Expression in Fibers Formed from Fast Muscle Myoblasts Is Not Due to the Absence of Innervation

An explanation for the lack of slow MyHC 2 gene expression in muscle fibers formed from PM myoblasts *in vitro* is that the cellular processes from the spinal cord explants did not make contacts with the muscle fibers and that no motor endplates formed to allow neuromuscular transmission. To examine this possibility, myoblasts from the MA and PM muscles of ED 12 chick embryos were cultured separately as before, and spinal cord explants were added to some of the cultures which were then incubated for an additional 7 days. At that time, rhodamine-conjugated α-bungarotoxin was added to all cultures to detect AchR clusters on muscle fibers. Cultures were fixed and immunostained with F59 and S58 (Fig. 7). Cultures without spinal cord explants did not contain AchR clusters (Figs. 7A–7D), nor were they immunostained by S58. All muscle fibers expressed a fast MyHC isoform(s) as demonstrated by staining with F59. In cocultures of spinal cord explants and muscle fibers formed from either MA or PM myoblasts, AchR clusters formed on the muscle fibers indicating that contact between the fibers and neuronal processes occurred in both culture types (Figs. 7E–7H). Although all muscle fibers immunostained with F59, only muscle fibers from MA myoblasts in coculture with spinal cord explants expressed the slow MyHC 2 gene and immunostained with S58 (Fig. 7G). Muscle fibers from fetal PM myoblasts cocultured with spinal cord explants contained AchR clusters but did not express the slow MyHC 2 gene (Fig. 7H). The results of these experiments demonstrate that the lack of slow MyHC2 gene expression in muscle fibers formed from fetal PM myoblasts was not due to a lack of nerve–muscle fiber contact.

Blockade of Neuromuscular Transmission or Depolarization Prevents Slow MyHC 2 Gene Expression

To determine whether neuromuscular transmission or membrane depolarization were required to initiate slow

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   1  AGTCCTATCACTAAGCGTTCAGGTGTCTGTCATTCTTCTTTTGCTGTTGCCAAGTCCTCC   60

  61  TCCAGCCAGACAAGATGTCTATGCTGGACATGAGCGAGTTTGGGGAGGCTGCTGAATACC  120
   1                M  S  M  L  D  M  S  E  F  G  E  A  A  E  Y  L   16

 121  TCCGGGAAAGCTACACAGAGCAGCTGAAGCGTCAGACGATCCCATTTGATGGGAAGAAGC  180
  17    R  E  S  Y  T  E  Q  L  K  R  Q  T  I  P  F  D  G  K  K  R   36

 181  GTGCTTGGATACCGGATGAGAAAGAAGCTTACATTGAAGTGGAAATCAAAGAAAGCACCG  240
  37    A  W  I  P  D  E  K  E  A  Y  I  E  V  E  I  K  E  S  T  G   56

 241  GTGGCAAAGTCACTGTGGAAACTAAAGATAAGCAAACACGGGTGGTGAAGGAGGATGAGC  300
  57    G  K  V  T  V  E  T  K  D  K  Q  T  R  V  V  K  E  D  E  L   76

 301  TGCAGCCCATGAACCCCCCCAAGTTCGACATGATCGAACACATGGCCATGCTGACGCATC  360
  77    Q  P  M  N  P  P  K  F  D  M  I  E  H  M  A  M  L  T  H  L   96

 361  TCAACGAGGCCTCCGTGCTCTACAACCTCAAGCGCCGCTATTCCCACTGGATGATCTACA  420
  97    N  E  A  S  V  L  Y  N  L  K  R  R  Y  S  H  W  M  I  Y  T  116

 421  CCTACTCGGGGCTCTTCTGCGTCACCATCAACCCCTACAAAGTGCTGCCAATCTACACTG  480
 117    Y  S  G  L  F  C  V  T  I  N  P  Y  K  V  L  P  I  Y  T  A  136

 481  CGCCCGTGGTGGCGGCCTACAAGGGGAAGCGGCGCTCTGAGGCTCCACCACACATCTACT  540
 137    P  V  V  A  A  Y  K  G  K  R  R  S  E  A  P  P  H  I  Y  S  156

 541  CCATCGCTGACAACGCCTACCAACGACATCTGCGCAACCGCGAGAACCAGTCCATGCTCA  600
 157    I  A  D  N  A  Y  Q  R  H  L  R  N  R  E  N  Q  S  M  L  I  176

 601  TCACCGGAGAATCTGGTGCTGGTAAGACTGTAAACACCAAGCGGGTCATTCAGTACTTTG  660
 177    T  G  E  S  G  A  G  K  T  V  N  T  K  R  V  I  Q  Y  F  A  196

 661  CCATTGTCGCAGCCTTGGGCGACACACCGGGCAAGAAATTAGCAGCTCTTGCCACTAAAA  720
 197    I  V  A  A  L  G  D  T  P  G  K  K  L  A  A  L  A  T  K  T  216

 721  CTGGGGGCACCCTCGAAGATCAAATCACTGAGGCTAACCCAGCTATGGAAGCTTTTGGCA  780
 217    G  G  T  L  E  D  Q  I  T  E  A  N  P  A  M  E  A  F  G  N  236

 781  ATGCCAAAACCATAAGGAATGACAACCCCTCACGTTTTGGCAAGGTTCATCGCATCCATT  840
 237    A  K  T  I  R  N  D  N  P  S  R  F  G  K  V  H  R  I  H  F  256

 841  TTGGCCCCTCAGGGAAGCTGGCCTCTGCGGCACATCGACACTCTACTCTTCTGCCAAAAT  900
 257    G  P  S  G  K  L  A  S  A  A  H  R  H  S  T  L  L  P  K  S  276

 901  CAAGAGTGATTTTCCAGCAACCCAAAGAGCGAAGCTACCATATCTACTACCAAATCCTCT  960
 277    R  V  I  F  Q  Q  P  K  E  R  S  Y  H  I  Y  Y  Q  I  L  S  296

 961  CTGGAAAGAAACCAGAGCTGCAAGACATGCTGCTGCTCTCCCTCAACCCCTACGATTACC 1020
 297    G  K  K  P  E  L  Q  D  M  L  L  L  S  L  N  P  Y  D  Y  H  316

1021  ACTTCTGCTCTCAGGGTGTAACAACTGTGGACAACTTGGATGATGGCGAGGAGCTCATGG 1080
 317    F  C  S  Q  G  V  T  T  V  D  N  L  D  D  G  E  E  L  M  A  336

1081  CAACAGATCATGCCATGGACATCCTGGGCTTCAGCAACGATGAGAAATACGGCTCCTATA 1140
 337    T  D  H  A  M  D  I  L  G  F  S  N  D  E  K  Y  G  S  Y  K  356

1141  AAATAGTGGGCGCTATCATGCACTTTGGCAACATGAAGTTCAAACAGAAGCAGCGGGAAG 1200
 357    I  V  G  A  I  M  H  F  G  N  M  K  F  K  Q  K  Q  R  E  E  376

1201  AGCAGGCAGAGGCTGACGGCACTGAAAGTGCTGACAAAGCTGCCTACCTCATGGGGATCA 1260
 377    Q  A  E  A  D  G  T  E  S  A  D  K  A  A  Y  L  M  G  I  S  396

1261  GCTCAGCTGACCTCATCAAGGGGCTGCTCCATCCTCGTGTGAAAGTGGGCAATGAGTACG 1320
 397    S  A  D  L  I  K  G  L  L  H  P  R  V  K  V  G  N  E  Y  V  416

1321  TGACCAAAGGTCAGAACGTGGAGCAGGTTGTCTATGCTGTGGGAGCCCTGGCTAAAGCCA 1380
 417    T  K  G  Q  N  V  E  Q  V  V  Y  A  V  G  A  L  A  K  A  T  436

1381  CTTATGATCGTATGTTCAAGTGGCTGGTCACTCGGATCAACAAGACCCTGGACACCAAGT 1440
 437    Y  D  R  M  F  K  W  L  V  T  R  I  N  K  T  L  D  T  K  L  456

1441  TGCCCAGGCAGTTCTTCATCGGAGTACTGGACATTGCAGGCTTCGAGATCTTTGATTTCA 1500
 457    P  R  Q  F  F  I  G  V  L  D  I  A  G  F  E  I  F  D  F  N  476

1501  ACAGCTTTGAGCAGCTGTGCATCAACTTTACTAATGAGAAGTTGCAGCAGTTCTTCAACC 1560
 477    S  F  E  Q  L  C  I  N  F  T  N  E  K  L  Q  Q  F  F  N  H  496

1561  ACCACATGTTTGTCCTGGAGCAAGAAGAATACAAGAAGGAAGGCATCGAATGGGTCTTCA 1620
 497    H  M  F  V  L  E  Q  E  E  Y  K  K  E  G  I  E  W  V  F  I  516

1621  TTGACTTTGGCTTGGACCTACAGGCTTGCATTGACCTGATTGAGAAGCCAATGGGAATCC 1680
 517    D  F  G  L  D  L  Q  A  C  I  D  L  I  E  K  P  M  G  I  L  536

1681  TGTCCATCCTCGAAGAGGAGTGCATGTTCCCCAAAGCCTCTGACATGTCGTTTAAAGCTA 1740
 537    S  I  L  E  E  E  C  M  F  P  K  A  S  D  M  S  F  K  A  K  556

1741  AGCTCTACGACAACCACCTTGGGAAGTCACCCAACTTCCAGAAACCCCGGCCGTCGAAGC 1800
 557    L  Y  D  N  H  L  G  K  S  P  N  F  Q  K  P  R  P  S  K  R  576

1801  GGC  1803
```

```
qSM2       MSMLDMSE  FGEAAEYLRE SYTEQLKRQT IP FDGKKRA WIPDEKEAYI EVEIKESTGG KVTVETKDKQ

qSM3           MEAL  L-A--PF--A PEGPH    SA P- G-TRGLC FV-HPQLEFV RAR-TARA-N G---T TETG

rcarβ       -ADRE-AA  --AG-PF--K -EK-R-EA-- R- --L--DV FV--D--EFV KAK-VSRE-- ---A- TENG
rcarα       -TDAQ-AD  --A -R---K -EK-R-EA-- R- --IRTEC FV--D--E-V KAK-VSRE-- ---A- TENG

cembf      MATDA--AI  -----P---K -EK-RIEA-N K- --A-SSV FVVHA--S-V KST-QSKES- ----K TEGG
cneof    M S-DAE-AI   -----P---K -EK-RIEA-N K- --A-TSV FVVHA--S-V KST-QSKES- ----K TEGG
cadtf    MASPDAE-AA   -----P---K -EK-RIEA-N K- --A-SSV FVVHP--SFV KGT-QSKE-- ----K TEGG

csmth          MSQKP LSDDEKF-FV DKNFVNNPLA QADWSAKKLV -V-S--HGFE AAS---EK-D E----LQENG



qSM2     TRV VKEDEL  QPMNPPKFDM IEHMAMLTHL NEASVLYNLK RRYSHWMIYT YSGLFCVTIN PYKVLPIYTA

qSM3     ETLT-P-ADV  H-Q------R --D-----F- H-PA------ E--AS----- --------V- ---W--V-N-

rcarβ    KVTV----QV  MQQ------K --D-----F- H-PA------ E--AS----- --------V- ---W--V-N-
rcarα    KVTV----QV  MQQ------K --D-----F- H-PA------ E--AA----- --------V- ---W--V-N-

cembf    ETLT----QI  FS-----Y-K --D---M--- H-PA------ E--AA----- --------V- ---W--V-NP
cneof    ETLT----QI  FS-----Y-K --D---M--- H-PA------ G--AA----- --------V- ---W--V-NP
cadtf    ETLT----QV  FS-----Y-K --D---M--- H-PA------ E--AA----- --------V- ---W--V-NP

csmth    KK-TLSK-DI  -K------SK V-D--E--C- ------H--R E--FSGL--- -------V-- ---Q----SE
```

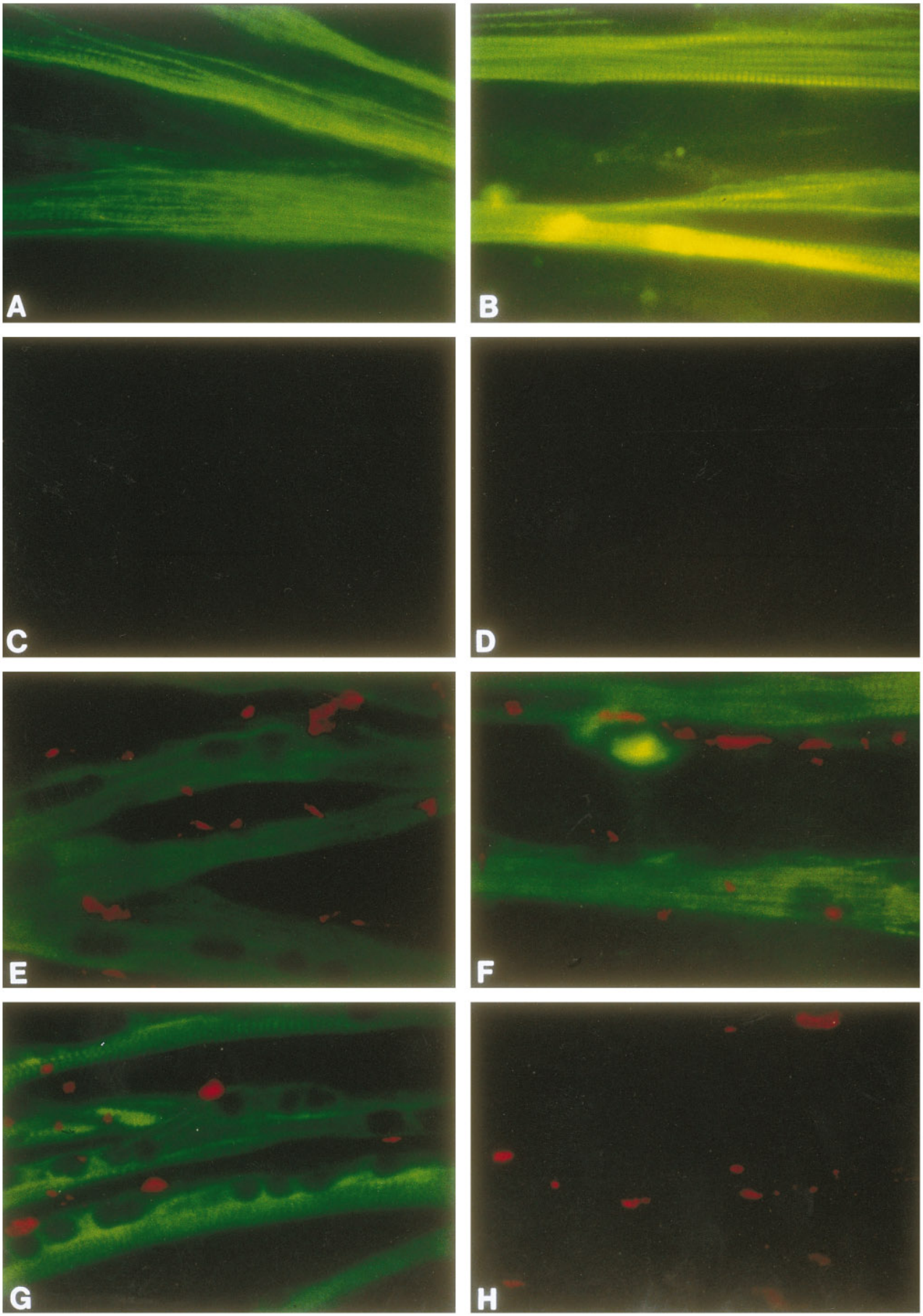
FIG. 6. Amino acid sequence comparison of slow MyHC 2 and other vertebrate MyHCs reveals regions of high sequence divergence. Comparison of the deduced amino acid sequence for slow MyHC 2 (qSM2) with other MyHCs shows a high degree of sequence divergence in the amino terminal 70 residues. The next region of 70 amino acids shares significantly higher sequence similarities. The other MyHCs include quail slow MyHC 3 (qSM3; Nikovits *et al.*, 1996), rat β-cardiac/slow and α-cardiac MyHCs (rcarb, rcara; McNally *et al.*, 1989), chicken embryonic, neonatal, and adult fast MyHCs (cembf, cneof, cadtf; Kropp *et al.*, 1986, 1987; Robbins *et al.*, 1986), and chicken smooth muscle MyHC (csmth; Yanagisawa *et al.*, 1987). Amino acid homologies between slow MyHC 2 and the other MyHC isoforms are represented by dashes.

MyHC 2 gene expression in cocultured muscle fibers, inhibitors of electrical transmission or inhibitors of the AchR were added to the cocultures. Tetrodotoxin (TTX) is an effective inhibitor of voltage-gated sodium channels, and its application inhibits pre- and postsynaptic depolarization. Addition of 10 μM TTX to the MA muscle–nerve cocultures resulted in an inhibition of the induction of slow MyHC 2 gene expression in muscle fibers (Fig. 8). Although all muscle fibers expressed a fast MyHC isoform(s) in the absence or presence of TTX as shown by F59 immunostaining (Figs. 8A and 8B), slow MyHC 2 gene expression did not occur in the presence of tetrodotoxin (Fig. 8C). *d*-Tubocurarine (curare) blocks neuromuscular transmission by inhibition of binding of acetylcholine to its receptor. Application of curare to cocultures of spinal cord explants and muscle fibers formed from MA myoblasts resulted in the absence of slow MyHC 2 gene expression (data not shown). This lack of expression of slow MyHC in the presence of tetrodotoxin or curare in cocultures demonstrates that the mere presence of spinal cord explants was not sufficient to induce slow MyHC 2 gene expression, and that electrical transmission or depolarization was required. Although these results do not completely rule out the possibility that cell–cell interactions mediated by diffusible factors other than acetylcholine could be involved in the induction of slow MyHC 2 gene expression in this coculture system, electrical transmission and functional AchRs are necessary components in the mechanism leading to slow MyHC 2 gene expression.

DISCUSSION

The difference between MyHC gene expression in fetal muscle fibers *in vivo* and *in vitro* appears to be produced by innervation with the important caveat that differences reflect the myoblast lineage from which the fibers form. When muscle fibers formed from fetal chick myoblasts were cultured in the presence of embryonic spinal cords containing motor neurons, expression of a slow MyHC gene was induced, but only in fibers formed from myoblasts of slow muscle origin. These experiments demonstrated that slow MyHC gene expression in fetal muscle fibers *in vitro*

FIG. 5. Nucleotide and deduced amino acid sequences of the slow MyHC 2 cDNA. This partial cDNA of 1803 bp contains an open reading frame of 1729 bp and encodes the amino terminal 576 amino acids of the gene. In addition it contains 74 bp of 5′ untranslated sequence. Three possible initiating methionines are located within the amino terminal six residues.


A
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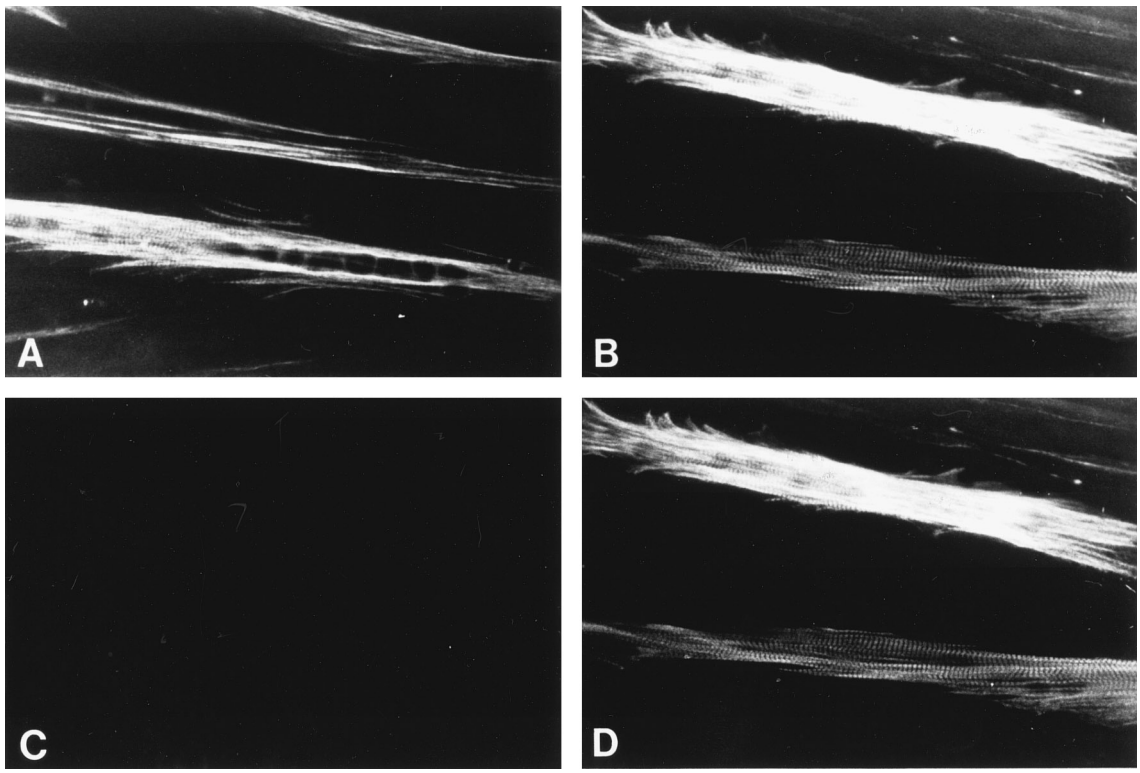

FIG. 8. Tetrodotoxin inhibits slow MyHC 2 gene expression in muscle–nerve cocultures. Fetal medial adductor myoblasts were plated as earlier and the resulting muscle fibers were cultured with spinal cord explants with (A, C) or without (B, D) 10 μM tetrodotoxin. After 10 days, cultures were immunostained with mAbs F59 (A, B) and S58 (C, D) to detect fast and slow MyHC isoforms. Tetrodotoxin effectively blocked induction of slow MyHC 2 gene expression in muscle fibers cocultured with spinal cord explants.

is subject to extrinsic regulation as well as intrinsic regulation by differences among myoblasts. Fiber type control is dependent on functional innervation because inhibitors of neuromuscular transmission or membrane depolarization—curare or tetrodotoxin—block the induction of expression of the slow MyHC 2 gene in muscle fibers in the presence of spinal cord explants. Therefore, the regulation of slow MyHC 2 gene expression and fiber type diversity in this *in vitro* system mimics secondary fiber formation *in vivo*.

The experimental results presented here provide a model system in which both intrinsic and extrinsic regulation of MyHC gene expression can be studied in an experimentally accessible *in vitro* design. Until now differentiation of fetal chick myoblasts *in vitro* did not reflect the patterns of MyHC gene expression and fiber type diversity seen during secondary muscle fiber formation *in vivo*. Mass cultures of fetal chick myoblasts from all muscles differentiated *in vitro* to form muscle fibers which expressed fast MyHC genes and did not express the slow MyHC 2 gene. Indeed, of 481 fetal myoblast clones tested, none differentiated into muscle fibers expressing slow MyHC. It was critical in developing a system to study the question of slow MyHC regulation by innervation to use fetal chick myoblast-derived fibers because other avian species, such as the quail, can contain fetal myoblasts that form muscle fibers that express slow MyHC even without innervation (Schafer *et al.*, 1987; Feldman and Stockdale, 1991). This is particularly

FIG. 7. Acetylcholine receptor (AchR) clusters appear on muscle fiber cultures derived from the medial adductor or pectoralis muscles with spinal cord explants. Muscle fibers from ED 12 medial adductor (A, C, E, G) and pectoralis (B, D, F, H) myoblasts were cultured in the absence (A–D) or presence (E–H) of ED 5 chick spinal cord explants. All cultures were treated with 100 n*M* rhodamine-conjugated α-bungarotoxin to detect AchRs. Cultures were double immunostained with mAbs F59 (A, B, E, F) and S58 (C, D, G, H) to detect fast and slow MyHC gene expression. Cultures without spinal cord explants exhibited little clustering of AchRs and did not express the slow MyHC 2 gene. Medial adductor myoblast-derived muscle fibers stained positively for AchR clusters and slow MyHC 2 only when cultured with spinal cord explants. Muscle fibers formed from pectoralis myoblasts did not express the slow MyHC 2 gene even though the presence of AchR clusters indicates they were in contact with neurites.

evident in long-term cultures of several weeks. For this reason, chick-derived muscle cultures were employed here rather than quail which have also been used to examine effects of innervation (Lefeuvre *et al.*, 1996).

Proteins of the MyHC family have highly conserved primary sequences which often results in varying amounts of cross-recognition of antibodies to specific slow MyHC isoforms, even when using monoclonal antibodies (Page *et al.*, 1992). Therefore it was necessary to generate slow MyHC isoform-specific nucleotide probes to determine which slow MyHC gene(s) was expressed in the MA/spinal cord cocultures. Slow MyHC 3-specific probes were already available, but none existed for slow MyHCs 1 and 2. Because DNA and deduced amino acid sequence identity decreases near the 5′ end of MyHC isoforms, this region was used to generate oligonucleotides specific for each slow myosin isoform. On Northern hybridization, each probe demonstrated the expected pattern of hybridization predicted from the patterns of protein expression that have been accepted to define each slow MyHC isoform during muscle development *in vivo*. These probes demonstrate conclusively that the slow MyHC gene which was upregulated in response to coculture with spinal cord segments is slow MyHC 2.

Nerve–muscle contacts in this coculture system resulted in the formation of AchR clusters. During myoblast cell cycle withdrawal and differentiation, AchR genes are upregulated in the absence of innervation (Buonanno and Merlie, 1986; Frail *et al.*, 1989), and the receptors are found dispersed throughout the plasma membrane. Upon innervation, the AchR genes are downregulated, except in subsynaptic nuclei where AchR mRNA levels remain high (Merlie and Sanes, 1985; Fontaine and Changeux, 1989). The receptors become localized to the neuromuscular junction by anchoring to agrin from motor neurons (Reist *et al.*, 1992). In the experiments presented here, only those muscle fibers cultured in the presence of spinal cord explants displayed abundant patches of AchRs. It is not known whether a single nerve–muscle contact is sufficient to induce slow MyHC 2 gene expression in the muscle fibers in these cocultures. It does not appear that expression of the slow MyHC 2 gene is restricted to the subsynaptic region like other innervation-dependent, muscle-specific genes (Merlie and Sanes, 1985; Sanes *et al.*, 1991), based on the uniform staining with S58 of the muscle fibers in coculture. Lack of slow MyHC 2 gene expression in fibers derived from PM myoblasts was not due to lack of neuromuscular junctions, since rhodamine-conjugated α-bungarotoxin staining revealed numerous AchR clusters on their surfaces. These findings mimic those *in vivo*, where the MA contains muscle fibers all of which come to express the slow MyHC 2 gene, whereas the PM contains fibers that only express fast MyHC genes. Differentiation of fetal myoblasts and fiber-type-specific expression of the slow MyHC 2 gene in both MA and PM secondary muscle fibers *in vivo* is appropriately recapitulated in the nerve–muscle coculture system.

Recent work of Lefeuvre *et al.* (1996) on cultures of quail ED 7 posterior latissimus dorsi (a fast muscle)-derived and anterior latissimus dorsi (a slow muscle)-derived myoblasts are at variance with these results. These workers used antibodies directed to slow myosins to identify the types of fibers formed from these two sources of myoblasts. Unlike the findings reported here, Lefeuvre and co-workers report that fibers formed in cell culture from either a fast or slow muscle source express all three slow MyHCs (slow MyHC1, 2 and 3) without innervation and that innervation only produces a quantitative change in the proteins expressed. The differences could be due to the use of fetal quail rather than chicken myoblasts which in long-term culture can express slow isoforms (Schafer *et al.*, 1987), to the use of early stages (ED 7) of avian muscle which could still contain some embryonic myoblasts which also produce slow MyHC in the absence of innervation (Miller and Stockdale, 1986b), or to the method employed to identify the slow myosins expressed.

Expression of slow MyHC isoforms has been detected in cultures of electrically chronically stimulated muscle fibers generated from satellite cells of the rat (Wehrle *et al.*, 1994). In such cultures varying percentages of muscle fibers express slow MyHC and the variation in expression appeared to depend on the muscle origin from which satellite cells were isolated. The highest percentage of slow MyHC-expressing muscle fibers was derived from slow muscle satellite cells. Such observations provide additional support for the contention that both intrinsic and extrinsic mechanisms regulate expression of slow MyHC in different muscle fiber types, and are in agreement with previous studies examining the relationship of innervation and MyHC gene expression both *in vivo* and *in vitro* (Condon *et al.*, 1990b; Crow and Stockdale, 1986b; McLennan, 1983; Wehrle *et al.*, 1994). This report extends these studies by definitively identifying and cloning the slow MyHC gene whose expression is induced by innervation and by characterizing the functional cell–cell relationship between muscle fibers and neurons in the induction of the slow MyHC 2 gene.

Expression of the slow MyHC 2 gene in muscle fibers formed from fetal PM myoblasts is intrinsically excluded from the subset of genes to be expressed, even when innervated in the coculture system described here. The intrinsically restricted potential of muscle fibers to express only a subset of MyHC genes has also been suggested from cross-reinnervation and transplantation studies in which regenerating muscle fibers expressed unique MyHC isoforms indicative of the muscle of origin rather than of the new source of innervation (Hoh and Hughes, 1988). Additionally, as described above, cross-reinnervation and denervation often demonstrates lack of complete fiber type transitions, indicating a fiber-specific intrinsic component in the regulation of MyHC gene expression. Taken together, evidence *in vivo* and *in vitro* suggests that muscle fiber type diversity, as defined by differences in MyHC gene expression, is controlled by both intrinsic (myoblast commitment) and extrinsic (cell–cell interactions) mechanisms. The experimental evidence also suggests that primary and secondary fiber myogenesis differs in the importance of these two mechanisms. Primary muscle fiber phenotype may principally be established by myoblast commitment to particular develop-

mental fates, while secondary fiber phenotype is regulated by myoblast commitment and innervation.

Intrinsic and extrinsic regulation of skeletal muscle fiber type diversification largely has been viewed as the independent mechanism that establishes and maintains phenotypic differences. In contrast, the results presented here indicate that intrinsic and extrinsic mechanisms of muscle fiber type formation are not separable mechanisms. The results presented here indicate that muscle fiber type formation is regulated by both intrinsic and extrinsic mechanisms (Stockdale, 1992, 1997). It is anticipated that an *in vitro* system in which both intrinsic and cell–cell interactive mechanisms are operative in defining muscle fiber type will provide opportunities to further characterize mechanisms that unite these processes.

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